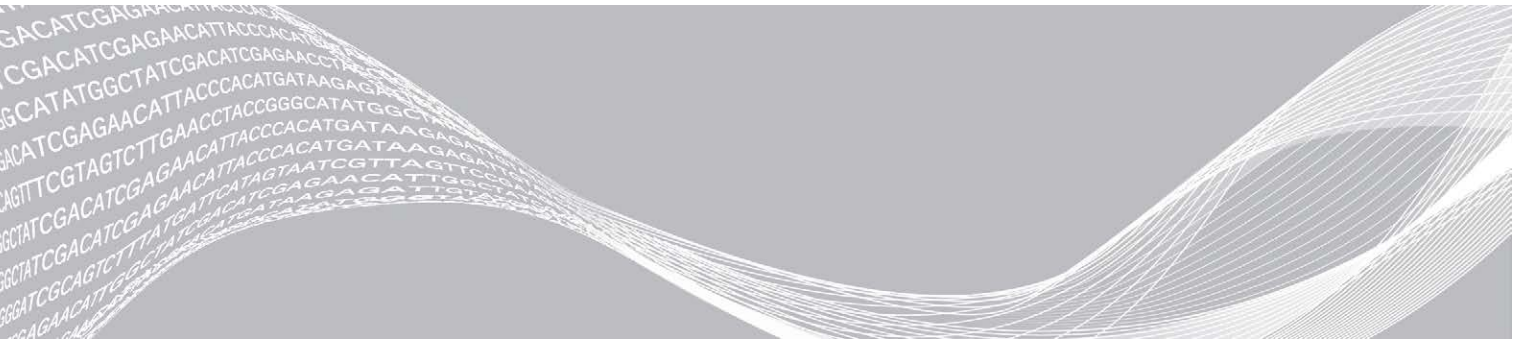
illumina®

Nextera™ DNA Flex Library Prep

Reference Guide



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For Research Use Only. Not for use in diagnostic procedures.

Revision History

Document	Date	Description of Change
Document # 1000000025416 v06	March 2019	Corrected PTC program thermal cycler temperature.
Document # 1000000025416 v05	March 2019	Corrected loading concentration values. Added information about separate workflow component requirements, DNA purity assessment, and about quantification and normalization of libraries. Added Local Run Manager Guide to additional resources. Added reagent overage information and PTC program settings to tagmentation step. Revised tagmentation amplification to include AMP program information and correct multichannel pipette volume. Revised workflow diagram to include RSB reagents. Revised cleanup step to include separate steps for small PCR amplicons and standard DNA input and to correct safe stopping storage days. Removed "Chapter 3 Sequencing." Added supplementary SPB step to Analytical Fragment Analyzer. Revised final loading concentrations in dilute libraries step. Added information about reagent storage temperature to ensure performance. Added new table with component and kit information and re-organized index kit information. Added Fragment Analyzer and Bioanalyzer to consumables table, added Fragment Analyzer and Agilent Technologies to equipment table, and added CD and UD acronyms.
Document # 1000000025416 v04	October 2018	Corrected average library size.
Document # 1000000025416 v03	October 2018	Updated Index Adapter terminology. Updated to include IDT® for Illumina®-Nextera™ DNA UD Indexes Set A (96 Indexes, 96 Samples). Updated diluting to starting concentration information. Added clarification in regards to ordering index adapters. Added additional resource information for Unique Dual Indexes. Added catalog number information for IDT® for Illumina®-Nextera™ DNA UD Indexes Set A (96 Indexes, 96 Samples) and Axygen® 1.7 mL MaxyClear Snaplock Microcentrifuge Tubes. Updated storage information for Lysis Reagent Kit. Clarified PCR Amplicons information. Clarified instructions when safe stopping is an option. Moved recommended read lengths for each system to the support site. Moved blood and lysis consumables to their own table. Revised step-by-step instructions to be more succinct. Reorganized the following content to improve continuity: • Rearranged DNA input recommendations. • Moved information on blood and saliva lysis preparation and procedures.

Document	Date	Description of Change
Document # 1000000025416 v02	June 2018	Added information about PCR Amplicons.
Document # 1000000025416 v01	April 2018	Replaced references to the *Nextera DNA Flex Pooling Guide (document # 1000000031471)* with the *Index Adapters Pooling Guide (document # 1000000041074)*. Pooling information is consolidated into the *Index Adapters Pooling Guide*.
Document # 1000000025416 v00	October 2017	Initial release.

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Chapter 1 Overview

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Introduction

This protocol explains how to prepare up to 96 dual-indexed paired-end libraries from DNA using the Nextera DNA Flex Library Prep workflow.

The workflow requires the following separate components:

- Nextera DNA Flex Library Prep
- IDT for Illumina–Nextera DNA Unique Dual (UD) Indexesor Nextera DNA Combinatorial Dual (CD) Indexes

The Nextera DNA Flex Library Prep workflow:

- Uses an enzymatic reaction, called tagmentation, to fragment DNA and add adapter sequences in only 15 minutes
- Innovates sample normalization at inputs ≥ 100 ng
- Streamlines sample pooling and sequencing
- Reduces excessive pipetting and overall hands-on time, while optimizing use of consumables by using master mix reagents
- Generates libraries from as little as 1 ng input
- Can prepare libraries directly from whole blood or saliva samples when using an optional accessory kit

DNA Input Recommendations

The Nextera DNA Flex Library Prep protocol is compatible with DNA inputs ranging from 1–500 ng, or higher. For human DNA samples and other large complex genomes, the recommended minimum DNA input is between 100–500 ng. For small genomes (eg microbial), the DNA input amount can be reduced to as low as 1 ng (modifying the PCR cycling conditions accordingly).

Assess DNA purity to make sure that the initial DNA sample does not contain > 1 mM EDTA and is free of organic contaminants, such as phenol and ethanol. These substances can interfere with the Nextera tagmentation reaction and result in unexpected library insert sizes.

DNA Input 100–500 ng

For DNA inputs between 100–500 ng, quantification of the initial DNA sample is not required, and normalization of the final yield is expected.

DNA Input < 100 ng

If using < 100 ng DNA input, quantification of the initial DNA sample to determine the number of PCR cycles required is recommended. Use a fluorometric-based method to quantify double-stranded DNA input. Avoid methods that measure total nucleic acid, such as NanoDrop or other UV absorbance methods. For more information see *Sample Input Recommendations* on page 2.

This protocol does not normalize final library yields from < 100 ng DNA input. Therefore, quantification and normalization of libraries before sequencing is required.

Assess DNA Purity

UV absorbance is a common method used for assessing the purity of a DNA sample. The ratio of absorbance at 260 nm to absorbance at 280 nm provides an indication of sample purity. This protocol is optimized for DNA with 260/280 absorbance ratio values of 1.8–2.0, which indicates a pure DNA sample. Target a 260/230 ratio of 2.0–2.2. Values outside this range indicate the presence of contaminants that interfere with tagmentation and adversely impacts the final library yield. For a complete list of contaminants, including sources, avoidance, and effects on the library preparation, see the *Nextera XT Troubleshooting Technical Note*.

CAUTION

Incomplete tagmentation caused by contaminants can result in library preparation failure, poor clustering, or low quality sequencing results.

Blood and Saliva Input Recommendations

The Nextera DNA Flex protocol is compatible with fresh whole blood (requires the Flex Lysis Reagent Kit) and saliva sample inputs. For information about protocols specific to blood and saliva, see *Blood Lysis* on page 17 or *Saliva Lysis* on page 19.

When starting with 10 µl of liquid whole blood in EDTA tubes or 30 µl of saliva in Oragene tubes, expect normalization of libraries equal to that observed when using ≥ 100 ng DNA input. Blood and saliva are heterogeneous sample types, therefore the ability of Nextera DNA Flex to generate normalized libraries depends on the total amount of DNA obtained from the lysed sample. The following factors can adversely affect normalization of library independent of kit performance:

- Viscosity of the saliva samples
- Blood sample age
- Storage conditions
- Underlying medical conditions affecting white blood cell counts

Sample Input Recommendations

The Nextera DNA Flex workflow is compatible with blood and saliva samples when using the following:

- Illumina Blood Lysis Protocol (blood) with Flex Lysis Reagent Kit
- Illumina Saliva Lysis Protocol (saliva)

The recommended PCR cycles for the BLT PCR program are adjusted based on sample input concentration and quality. For more information, see *Amplify Tagmented DNA* on page 9.

Table 1 DNA Input Recommendations

Total DNA Input (ng)	Quantification of Input DNA Recommended	Normalized Library Yield
1–9	Yes	No
10–24		
25–49		
50–99		
100–500	No	Yes
Blood/Saliva	No	Yes

PCR Amplicons

When starting with PCR amplicons, the PCR amplicon must be > 150 bp. The standard clean up protocol depletes libraries < 500 bp. Therefore, Illumina recommends that amplicons < 500 bp undergo a 1.8 x sample purification bead volume ratio to supernatant during *Clean Up Libraries* on page 11. Shorter amplicons can otherwise be lost during the library cleanup step.

Tagmentation cannot add an adapter directly to the distal end of a fragment, so a drop in sequencing coverage of ~50 bp from each distal end is expected. To ensure sufficient coverage of the amplicon target region, design primers to extend beyond the target region by 50 bp per end.

Additional Resources

The *Nextera DNA Flex Library Prep support page* on the Illumina website provide additional resources. These resources include software, training, compatible products, best practices, and the following documentation. Always check the support pages for the latest versions.

Resource	Description
Custom Protocol Selector	A wizard for generating end-to-end documentation customized for the library prep method, run parameters, and analysis method used for the sequencing run.
Nextera™ DNA Flex Library Prep Checklist (document # 1000000033561)	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
Nextera™ DNA Flex Library Prep Consumables and Equipment List (document # 1000000033564)	Provides an interactive checklist of user-provided consumables and equipment.
Index Adapter Pooling Guide (document # 1000000041074)	Provides pooling guidelines and dual indexing strategies for using the 10 base pair IDT for Illumina-Nextera DNA UD Indexes or 8 base pair Nextera CD Indexes with the Nextera DNA Flex Library Prep workflow.
Illumina Adapter Sequences (document # 1000000002694)	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.
Unique Dual Index Support page	Provides additional resources and information about Unique Dual Indexes and how to set up your instrument for sequencing runs with when using 10 base pair index adapters.
Illumina Experiment Manager Guide (document # 15031335)	Provides information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
Local Run Manager Software Guide (document # 1000000002702)	Provides an overview of the Local Run Manager software, instructions for using software features, and instructions for installing analysis modules on the instrument computer.
BaseSpace Sequence Hub Online Help	Information about the BaseSpace® sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.
Illumina Free Adapter Blocking Reagent (document # 1000000047585)	Provides protocol to block excess free adapter, minimize potential index hopping levels, and enhance data quality.

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Introduction

This chapter describes the Nextera DNA Flex Library Prep protocol.

- Review training videos before proceeding. See the training section on the *Nextera DNA Flex Library Prep support page*.
- Before proceeding, confirm kit contents and make sure that you have the required equipment and consumables. See *Consumables and Equipment* on page 26.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.

Prepare for Pooling

If you plan to pool libraries, record information about your samples before beginning library prep. For more information about Illumina Experiment Manager (IEM), Local Run Manager, or BaseSpace Prep Tab, see the *Nextera DNA Flex Library Prep support page*.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples or reagent master mixes, change tips between ***each sample***.
- When adding index adapters with a multichannel pipette, change tips between ***each row*** or ***each column***. If using a single channel pipette, change tips between each sample.
- If using index adapter tubes, only open one index adapter at a time to prevent misplacing caps. Remove unused index adapter tubes from the working area.

Preparing Nextera Dual Index Adapter Plate (UD or CD)

- Each index well is for single use only.

Prepare Nextera dual index adapter plate as follows.

- Centrifuge at 1000 x g for 1 minute to settle liquid away from the seal.
- If processing < 96 samples, pierce the foil seal on the index adapter plate with a new pipette tip for each well for only the number of samples being processed.
- If processing 96 samples, align a new Eppendorf 96-well PCR plate above the index adapter plate and press down to puncture the foil seal on all 96 wells.

Press the plate down slowly to avoid tipping the volume over.

- Discard the empty Eppendorf plate used to puncture the foil seal.

Sealing the Plate

- Always seal the 96-well plate with the adhesive seal using a rubber roller to cover the plate before the following steps in the protocol:
  - Shaking steps
  - Thermal cycling steps
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Microseal 'B' seals can be used for thermal cycling or short-term storage.
- Microseal 'F' adhesive foils are effective at temperatures down to -70°C and are recommended for long-term storage of the 96-well plates containing the final libraries.

Handling Bead-Linked Transposomes (BLT)

- Store the BLT stock tube upright in the refrigerator so that the beads are always submerged in the buffer.
- Vortex the BLT stock tube thoroughly until the beads are resuspended. To avoid resettling the beads, centrifugation before pipetting is not recommended.
- If beads are adhered to the side or top of a 96-well plate, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.
- When washing beads:
  - Use the appropriate magnetic stand for the plate.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand.
  - Do not disturb the bead pellet.
  - If beads are aspirated into pipette tips, dispense back into the plate on the magnetic stand and wait until the solution is clear (~2 minutes).
  - Dispense tagment wash buffer (TWB) directly onto the beads.
  - If liquid becomes adhered to the side or top of the tube or well, centrifuge at 280 × g for 3 seconds to pull volume into solution.

Handling Tagment Wash Buffer (TWB)

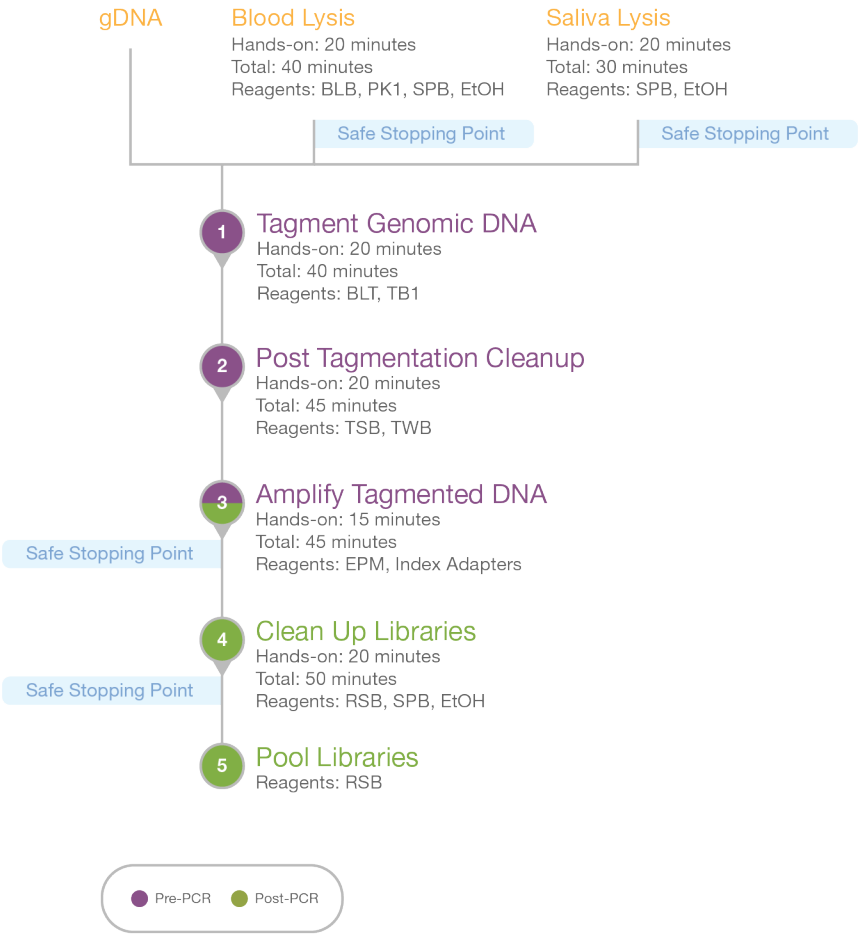
- Pipette slowly to minimize foaming.

Nextera DNA Flex Library Prep Workflow

The following diagram illustrates the Nextera DNA Flex Library Prep workflow. Safe stopping points are marked between steps.

Time estimates are based on preparing 16 samples using a multichannel pipette.

Figure 1 Nextera DNA Flex Library Prep Workflow



Tagment Genomic DNA

This step uses the Bead-Linked Transposomes (BLT) to tagment DNA. This process fragments and tags the DNA with adapter sequences.

Consumables

- BLT (Bead-Linked Tansposomes)
- TB1 (Tagmentation Buffer 1)
- Nuclease-free water
- 96-well PCR plate
- Microseal 'B' adhesive seal
- 1.7 ml microcentrifuge tubes
- 8-tube strip
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes

About Reagents

- BLT must be stored at temperatures above 2°C. Do not use BLT that has been stored below 2°C.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
BLT	2°C to 8° C	Bring to room temperature. Vortex to mix. Do not centrifuge before pipetting.
TB1	-25°C to -15°C	Bring to room temperature. Vortex to mix.

2 Save the following TAG program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- Step 1: 55°C for 15 minutes
- Step 2: Hold at 10°C

Procedure

1 Add 2–30 µl DNA to each well of a 96-well PCR plate so that the total input amount is 100–500 ng.

2 If DNA volume < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.

3 Vortex BLT vigorously for 10 seconds to resuspend. Repeat as necessary.

4 Combine the following volumes to prepare the tagmentation master mix. The volume required per reaction is:
- BLT (11 µl)
- TB1 (11 µl)

Reagent overage is included in the volume to ensure accurate pipetting.

5 Vortex the tagmentation master mix thoroughly to resuspend.

6 Divide the tagmentation master mix volume equally into an 8-tube strip.

7 Using fresh tips for each sample column, with a 20 µl multichannel pipette, transfer 20 µl tagmentation master mix to each well of the plate containing a sample. Pipette to resuspend.
Discard the 8-tube strip once the tagmentation master mix has been dispensed.

8 Seal the plate with Microseal 'B', place on the preprogrammed thermal cycler, and run the TAG program.

Post Tagmentation Cleanup

This step washes the adapter-tagged DNA on the BLT before PCR amplification.

Consumables

- TSB (Tagment Stop Buffer)
- TWB (Tagment Wash Buffer)
- 96-well plate magnet
- Microseal 'B' adhesive seal
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
TSB	15°C to 30°C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Use at room temperature.
TWB	15°C to 30°C	Use at room temperature.

2 Save the following PTC program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 60 µl
- Step 1: 37°C for 15 minutes
- Step 2: Hold at 10°C

Procedure

1 Add 10 µl TSB to the tagmentation reaction.

2 Slowly pipette each sample well 10 times to resuspend the beads.

3 Seal the plate with Microseal 'B', place on the preprogrammed thermal cycler, and run the PTC program.

4 Place the plate on the magnetic stand and wait until solution is clear (~3 minutes).

5 Using a multichannel pipette, remove and discard supernatant.

6 Wash two times as follows:

NOTE

Minimize the potential of TWB foaming during the tagmentation wash by using a deliberately slow pipetting technique to avoid incorrect volume aspiration and incomplete mixing.

a Remove the sample plate from the magnetic stand and add 100 µl TWB directly onto the beads.
b Pipette slowly until beads are fully resuspended.
c Place the plate on the magnetic stand and wait until the solution is clear (~3 minutes).
d Using a multichannel pipette, remove and discard supernatant.

7 Remove the plate from the magnetic stand and add 100 µl TWB.

8 Pipette each sample well slowly to resuspend the beads.

9 Seal the plate and place on the magnetic stand until the solution is clear (~3 minutes). Keep on the magnetic stand until step 4 of the *Procedure* section in *Amplify Tagmented DNA*.
The TWB remains in the wells to prevent overdrying of the beads.

Amplify Tagmented DNA

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation.

See the *Index Adapters Pooling Guide (document # 1000000041074)* for information to confirm that indexes selected for low plexity pooling have the appropriate color balance.

The index adapter tubes or plate is ordered separately from the library prep components. For a list of compatible index adapters for use with this protocol, see *Index Kit Contents* on page 25.

Consumables

- EPM (Enhanced PCR Mix)
- Index adapters (tubes or plate)
- Nuclease-free water
- Microseal 'B' adhesive seal
- 1.7 ml microcentrifuge tubes
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
EPM	-25°C to-15°C	Thaw on ice. Invert to mix, then briefly centrifuge.
Index Adapters (tubes or plate)	-25°C to-15°C	Thaw at room temperature. For index tubes: Vortex to mix, then centrifuge briefly. For index adapter plates: Spin briefly before use.

2 Save the following BLT PCR program on a thermal cycler using the appropriate number of PCR cycles, which are listed in the following table.
   - Choose the preheat lid option and set to 100°C
   - Step 1: 68°C for 3 minutes
   - Step 2: 98°C for 3 minutes
   - Step 3: (X) cycles of:
     - 98°C for 45 seconds
     - 62°C for 30 seconds
     - 68°C for 2 minutes
   - Step 4: 68°C for 1 minutes
   - Step 5: Hold at 10°C

Total DNA Input (ng)	Number of PCR Cycles (X)
1–9	12
10–24	8
25–49	6
50–99	5
100–500	5
Blood/Saliva	5

Procedure

1 Combine the following to prepare the PCR master mix. The volume required per reaction is:
   - EPM (22 µl)
   - Nuclease-free water (22 µl)

   Reagent overage is included in the volume to ensure accurate pipetting.

2 Vortex and centrifuge the PCR master mix at 280 × g for 10 seconds.

3 With the plate on the magnetic stand, use a 200 µl multichannel pipette to remove and discard supernatant.
   Foam that remains on the well walls does not adversely affect the library.

4 Remove from the magnet.

5 Immediately add 40 µl PCR master mix directly onto the beads in each sample well.

6 Immediately pipette to mix until the beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

7 Seal the sample plate and centrifuge at 280 × g for 3 seconds.

8 Add the appropriate index adapters to each sample.
   For tubes, open only one index adapter tube at a time to prevent misplacing caps; alternatively, use fresh caps after opening each tube.
   For plates, a well may contain >10 µl of index adapters. Do not add samples to the index adapter plate. Each well of the index plate is for a single use only.

Index Kit Type	Kit Configuration	Volume of Index Adapter per Sample
24 plex (dual index)	Individual tubes	5 µl i5 adapter 5 µl i7 adapter
96 plex (dual index)	96-well plate	10 µl pre-paired i5 and i7 index adapters

9 Using a pipette set to 40 µl, pipette 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

10 Seal the plate with Microseal 'B', and centrifuge at 280 × g for 30 seconds.

11 Place on the thermal cycler and run the BLT PCR program.

12 Remove the plate from the thermal cycler when the PCR program completes.

13 Centrifuge at 280 × g for 1 minute to collect contents at the bottom of the well.

SAFE STOPPING POINT

If you are stopping, store at 2°C to 8°C for up to 3 days.

Clean Up Libraries

This step purifies the amplified libraries through a double-sided bead purification procedure.

Consumables

- SPB (Sample Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- 96-well 0.8 ml Polypropylene Deepwell Storage Plate (midi plate) (2)
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Microseal 'F' foil seals
- 1.7 ml microcentrifuge tubes
- Nuclease-free water

NOTE

Use Microseal 'F' when sealing the plate for long-term storage. Use Microseal 'B' for other steps that require a sealed plate or long-term storage.

About Reagents

- SPB
  - Must be at room temperature before use
  - Vortex before each use
  - Vortex frequently to make sure that beads are evenly distributed
  - Aspirate and dispense slowly due to the viscosity of the solution

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
SPB	2°C to 8°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
RSB	-25°C to -15°C	Thaw and bring to room temperature. Vortex to mix.

Procedure

1 Place the plate on the magnetic stand and wait until the solution is clear (~5 minutes).

2 Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of a new midi plate.

3 Vortex and invert SPB multiple times to resuspend.

4 If you are using standard DNA input, do as follows.

   a Add 40 µl nuclease-free water to each sample well containing supernatant.
   b Add 45 µl SPB to each sample well containing supernatant.
   c Pipette each sample well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
   d Seal the plate and incubate at room temperature for 5 minutes.
   e Place on the magnetic stand and wait until the solution is clear (~5 minutes).
   f During incubation, thoroughly vortex the SPB (*undiluted* stock tube), and then add 15 µl to each well of a *new* midi plate.
   g Transfer 125 µl supernatant from each well of the first plate into the corresponding well of the second plate (containing 15 µl *undiluted* SPB).
   h Pipette each sample well in the second plate 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
   i Discard the first plate.

5 If you are using small PCR amplicons sample input, do as follows.

   a Add 81 µl SPB to each midi plate well containing supernatant.
   b Pipette each sample well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

6 Incubate the sealed midi plate at room temperature for 5 minutes.

7 Place on the magnetic stand and wait until the solution is clear (~5 minutes).

8 Without disturbing the beads, remove and discard supernatant.

9 Wash two times as follows.

   a With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
   b Incubate for 30 seconds.
   c Without disturbing the beads, remove and discard supernatant.

10 Use a 20 µl pipette to remove and discard residual EtOH.

11 Air-dry on the magnetic stand for 5 minutes.

12 Remove from the magnetic stand and add 32 µl RSB to the beads.

13 Pipette to resuspend.

14 Incubate at room temperature for 2 minutes.

15 Place the plate on the magnetic stand and wait until the solution is clear (~2 minutes).

16 Transfer 30 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

Pool Libraries

When the DNA input is 100-500 ng, quantifying and normalizing individual libraries generated in the same experiment is not necessary. However, the final yield of libraries generated in separate experiments can vary slightly.

To achieve optimal cluster density, pool equal library volumes and quantify the pool before sequencing.

DNA Inputs of 100–500 ng

1 Combine 5 µl each library (up to 96 libraries) in a 1.7 ml microcentrifuge tube.

2 Vortex to mix, and then centrifuge.

3 Quantify the library pool using a dsDNA fluorescent dye method, such as Qubit or PicoGreen.

For DNA Inputs of < 100 ng

1 Quantify each library individually using Qubit or PicoGreen.

Check Library Quality (Optional)

1 Run 1 µl library or pooled libraries on one of the following instruments:
- Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit.
  - Add 1 µl RSB to the library to achieve the 2 µl volume required for Fragment Analyzer.
- Agilent 2100 Bioanalyzer with a High Sensitivity DNA kit.

The following figures show typical library size profiles with an average fragment size of 600 bp when analyzed with a size range of 150–1500 bp.

Figure 2 Example Fragment Analyzer Trace

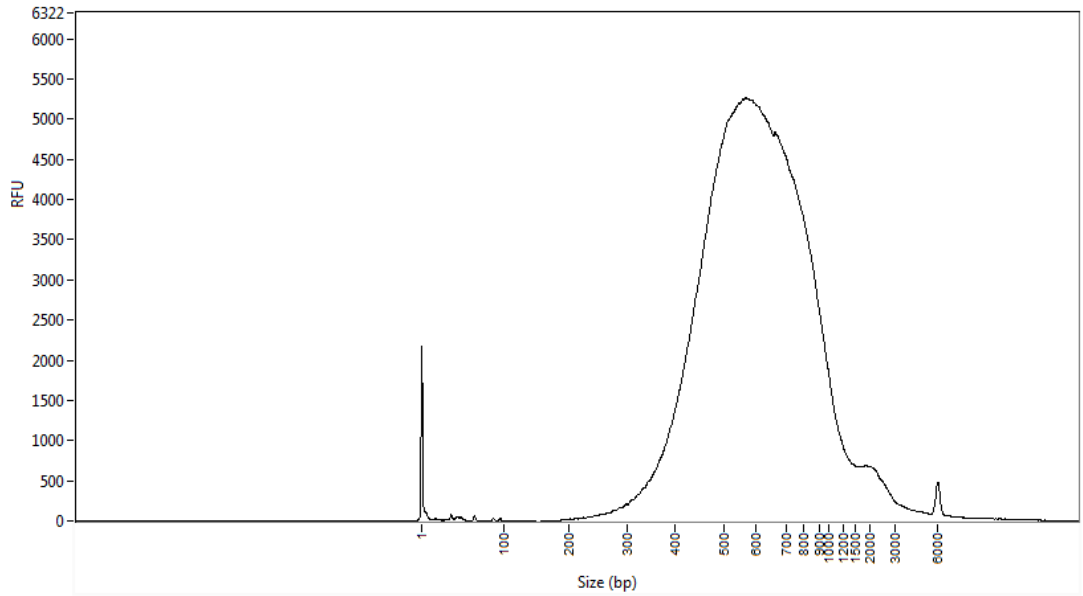
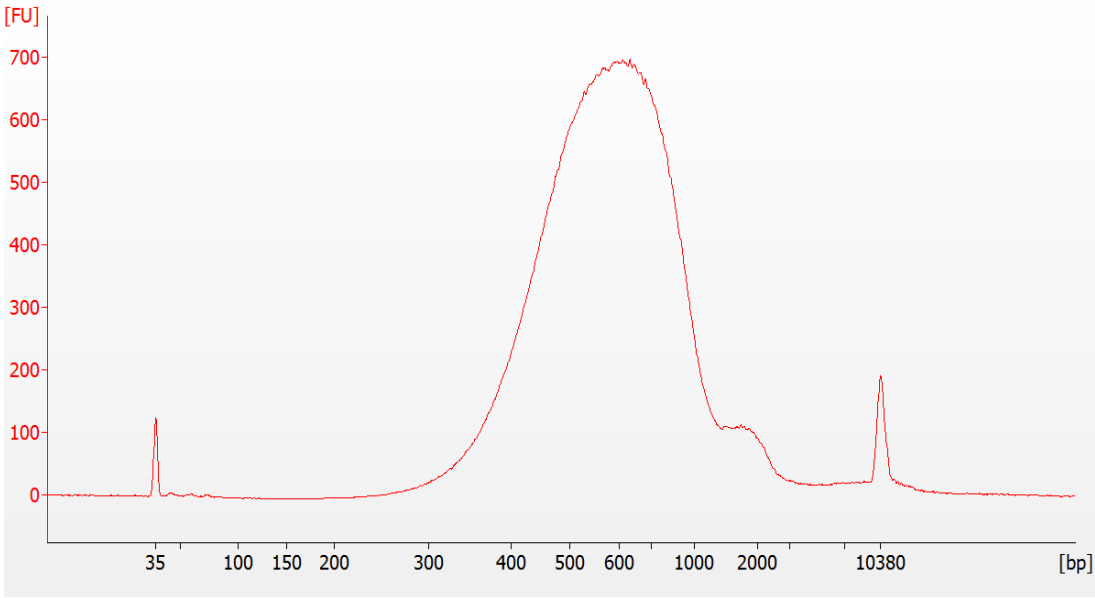


Figure 3 Example Bioanalyzer Trace



Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, Illumina recommends the following read lengths:

Table 2 Recommended Read Length on Illumina Systems

Sequencing System	Read Length
NovaSeq 6000, HiSeq X*, HiSeq 3000 and HiSeq 4000, NextSeq 500 and NextSeq 550, MiSeq, MiniSeq, iSeq 100	2 x 151
HiSeq 2000, HiSeq 2500 (high output)	2 x 126
HiSeq 2500 (rapid run)	2 x 101**

*Not compatible with IDT for Illumina-Nextera DNA UD Indexes (10bp)

**Assumes use of the 200 cycle kit.

If you are using IDT for Illumina-Nextera DNA UD Indexes, it is important to know that they use 10 base pair index codes that differ from the Nextera DNA CD Indexes, which use 8 base pair index codes. This change in base pair index codes can require adjustments to your sequencing run set up. For information on setup, see https://www.illumina.com/uniquedualindex.

1 Calculate the molarity value of the library or pooled libraries using the following formula.
   - For libraries qualified on a Bioanalyzer, use the average size obtained for the library.
   - For all other qualification methods, use 600 bp as the average library size.

$$\frac{ng/\mu l \times 10^6}{660\frac{g}{mol} \times average\ library\ size\ (bp)} = Molarity\ (nM)$$

2 Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
HiSeq 2500 and HiSeq 2000 (high output modes)	2	12
HiSeq 2500 (rapid run mode)	2	8.5
HiSeq X, HiSeq 4000, and HiSeq 3000	2–3	200-300
iSeq 100	2	200
MiniSeq	2	1.2–1.3
MiSeq (v3 reagents)	4	12
NextSeq 550 and NextSeq 500	2	1.2–1.3
NovaSeq 6000	2	See document # 1000000019358 (*NovaSeq 6000 System Guide*)

3 Dilute libraries using RSB:
   - **Libraries quantified as a multiplexed library pool**—Dilute the pool to the starting concentration for your system.
   - **Libraries quantified individually**—Dilute each library to the starting concentration for your system. Add 10 µl each diluted library to a tube to create a multiplexed library pool.

4 Follow the denature and dilute instructions for your system, diluting to the final loading concentration listed in the preceding table.
   - For the iSeq 100 System, see the system guide for dilution instructions (libraries are automatically denatured).
   - For the NovaSeq 6000 System, see the system guide for pool and denature instructions.
   - For the HiSeq 4000 and HiSeq 3000 Systems, see the cBot 2 or cBot system guide for reagent preparation instructions.
   - For all other systems, see the denature and dilute libraries guide.

   The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Appendix A Supplemental Procedures

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Saliva Lysis 19

Introduction

The contents of this section provide instructions for optional procedures within the Nextera DNA Flex Library Prep workflow.

Blood Lysis

Use this protocol when performing the Nextera DNA Flex Library Prep workflow using blood sample inputs with the Flex Lysis Reagent Kit. This protocol has been validated using fresh whole blood collected in EDTA collection tubes. Store the blood at 4°C and process it within 3 days.

This protocol is expected to generate > 100 ng of DNA output at the end of the blood lysis step.

NOTE

The use of frozen blood has not been validated and therefore cannot be recommended.

CAUTION

Blood is a potential source of infectious diseases. Follow site-specific procedures to ensure the safe handling of blood samples. During the lysis protocol, make sure that the entire blood sample is fully lysed (brown in color following the heat incubation step) before proceeding to subsequent steps.

Consumables

- EDTA collection tubes (for blood sample collection)
- SPB (Sample Purification Beads)
- BLB (Blood Lysis Buffer)
- PK1 (Proteinase K)
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plate

About Reagents

- SPB
  - Is included in the Nextera DNA Flex Library Prep Kit
  - Must be at room temperature before use
  - Vortex before each use
  - Vortex frequently to make sure that beads are evenly distributed
  - Aspirate and dispense slowly due to the viscosity of the solution

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
BLB	15°C to 30°C*	If frozen, thaw at room temperature. If precipitates are observed, heat at 37°C for 10 minutes and vortex until resuspended.
SPB	2°C to 8°C**	Let stand for 30 minutes to bring to room temperature.
PK1	-25°C to -15°C	Place on ice until needed.

*BLB is shipped -25°C to -15°C but stored at 15°C to 30°C.

**SPB is included in the Nextera DNA Flex Library Prep Kit.

2 Save the following BLP program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- Step 1: 56°C for 10 minutes

Procedure

1 Prepare a lysis master mix. For each reaction use:
- BLB (7 µl)
- PK1 (2 µl)
- Nuclease-free water (31 µl)

Reagent overage is included in the volume to ensure accurate pipetting.

2 Invert the EDTA tube 10 times to mix.

3 Transfer 10 µl blood from the tube to one well of a 96-well PCR plate.

4 Vortex and centrifuge the lysis master mix.

5 Add 40 µl lysis master mix to each sample.

6 Vortex and invert SPB multiple times to resuspend.

7 Add 20 µl SPB to the sample well.

8 Using a pipette set to 50 µl, slowly pipette 10 times to mix.

9 Seal the plate, place on the preprogrammed thermal cycler, and run the BLP program.

10 Place on a magnetic stand and wait 5 minutes.
The solution will not become clear due to the dark brown color of the blood from the lysis reaction. The beads migrate after 5 minutes.

11 Without disturbing the beads, remove and discard supernatant.

12 If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the solution is clear (~2 minutes).

13 Add 150 µl of fresh 80% EtOH to the well.

14 Pipette to remove all EtOH.

15 Incubate on the magnetic stand for 30 seconds.

16 Use a 20 µl pipette to remove and discard all residual EtOH.

17 Remove the plate from the magnetic stand.

18 Add 30 µl nuclease-free water and pipette to resuspend.

19 Proceed immediately to step 3 of *Tagment Genomic DNA Procedure* on page 7 or stop and store the sample bead mixture.

SAFE STOPPING POINT

If you are stopping before proceeding to *Tagment Genomic DNA* on page 7, seal the plate with a Microseal 'B' adhesive seal, and store the sample bead mixture at 2°C to 8°C for up to 3 days.

Saliva Lysis

Use this protocol when performing the Nextera DNA Flex Library Prep workflow using saliva sample inputs. This protocol is validated for saliva collected only in Oragene DNA Saliva collection tubes. The saliva is mixed with the Oragene DX Solution contained in the collection tube, making it stable at room temperature.

This protocol is expected to generate > 100 ng of DNA output at the end of the saliva lysis step.

WARNING

Saliva is a potential source of infectious diseases. Follow site-specific procedures to ensure the safe handling of saliva samples.

Consumables

- Oragene DNA collection tubes (for saliva sample collection)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plate

About Reagents

- SPB
  - Is included in the Nextera DNA Flex Library Prep kit
  - Must be at room temperature before use
  - Vortex before each use
  - Vortex frequently to make sure that beads are evenly distributed
  - Aspirate and dispense slowly due to the viscosity of the solution

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
Saliva samples in Oragene DNA collection tubes	Room temperature	Any time after sample collection, incubate for a minimum of 1 hour at 50°C in a water bath or an air incubator (as recommended by DNA Genotek) to lyse the cells. Following heat treatment, store at room temperature. For information on long-term storage of Oragene/saliva samples at room temperature and guarantees, see the DNA Genotek website.
SPB	2°C to 8°C*	Let stand for 30 minutes to bring to room temperature.

*SPB is included in the Nextera DNA Flex Library Prep Kit.

Procedure

1 For each sample, add 20 µl nuclease-free water to one well of a 96-well PCR plate.

2 Vortex the heat-treated Oragene DNA collection tube.

3 Transfer 30 µl saliva sample from the tube to the well containing water. Slowly pipette to mix. For viscous samples, use a wide-bored pipette tip for more accurate pipetting.

4 Vortex and invert SPB multiple times to resuspend.

5 Add 20 µl SPB to the sample well.

6 Using a pipette set to 50 µl, slowly pipette 10 times to mix.

7 Incubate at room temperature for 5 minutes.

8 Place on a magnetic stand and wait 5 minutes.

9 Without disturbing the beads, remove and discard supernatant.

10 If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).

11 Dispense 150 µl fresh 80% EtOH onto the SPB pellet and incubate for 30 seconds.

12 Incubate on the magnetic stand for 30 seconds.

13 Pipette to remove and discard the EtOH.

14 Use a 20 µl pipette to remove and discard all residual EtOH.

15 Remove the plate from the magnetic stand.

16 Add 30 µl nuclease-free water and pipette to resuspend.

17 Proceed immediately to step 3 of *Tagment Genomic DNA Procedure* on page 7 or stop and store the sample bead mixture.

SAFE STOPPING POINT

If you are stopping before proceeding to *Tagment Genomic DNA* on page 7, seal the plate with a Microseal 'B' adhesive seal, and store the sample bead mixture at 2°C to 8°C for up to 3 days.

Supporting Information

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How the Nextera DNA Flex Assay Works 22
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Consumables and Equipment 26
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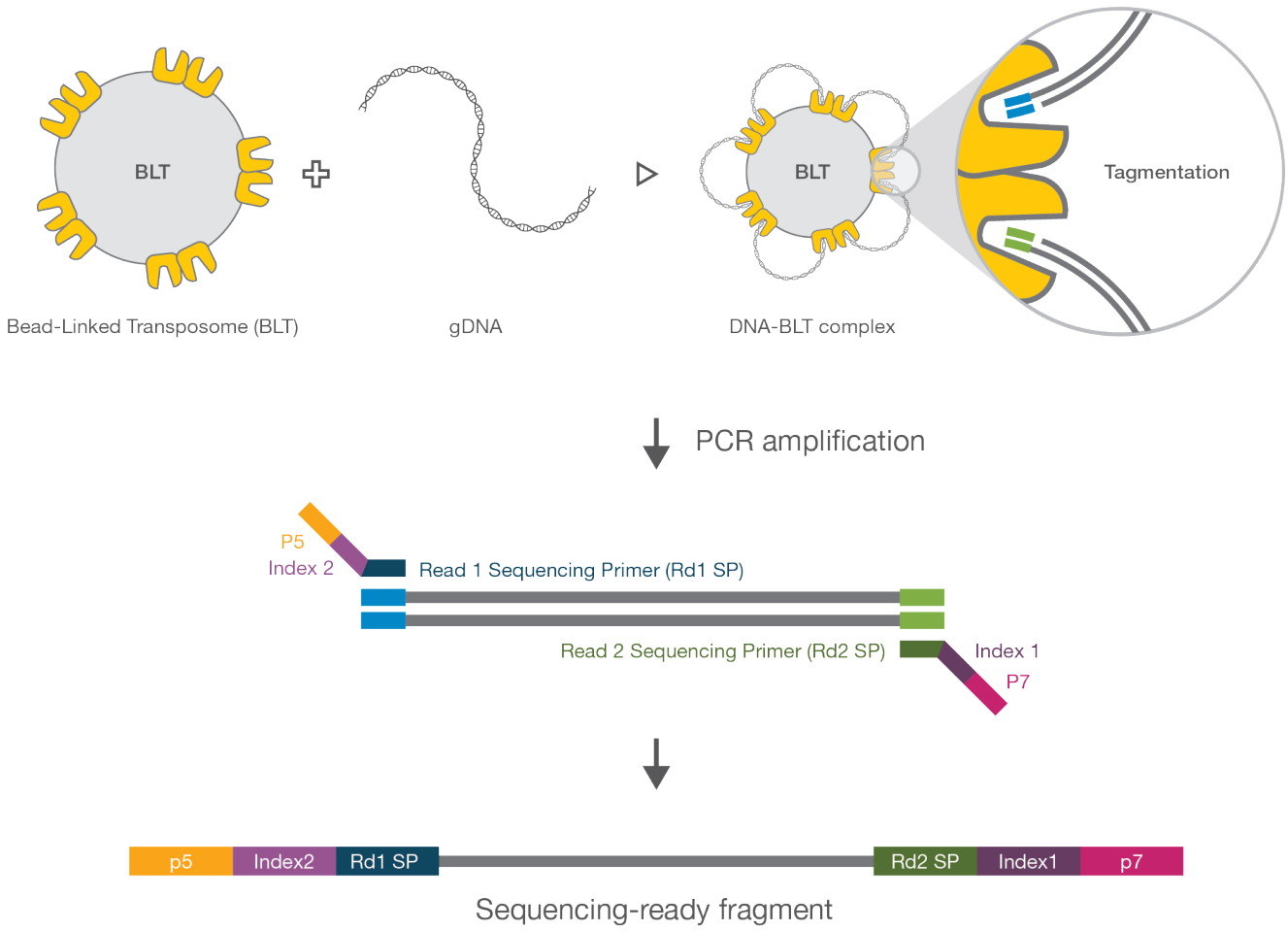
Introduction

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed workflow contents, and obtained all required consumables and equipment.

How the Nextera DNA Flex Assay Works

The Nextera DNA Flex library prep kit uses an innovative, bead-based transposome complex to tagment genomic DNA. This tagmentation is done by fragmenting and adding adapter tag sequences in a single reaction step. After it is saturated with input DNA, the bead-based transposome complex fragments a set number of DNA molecules. This fragmentation provides flexibility to use a wide DNA input range to generate normalized libraries of consistent tight fragment size distribution. Following tagmentation, a limited-cycle PCR step adds Nextera DNA Flex-specific index adapter sequences to the ends of a DNA fragment. This step enables capability across all Illumina sequencing platforms. A subsequent Sample Purification Beads (SPB) cleanup step then purifies libraries for use on an Illumina sequencer. The double-stranded DNA library is denatured before hybridization of the biotin probe oligonucleotide pool.

Figure 4 Nextera DNA Flex Workflow



Product Contents

The Nextera DNA Flex workflow requires the following components:

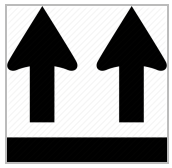
- Library Prep
- Indexes

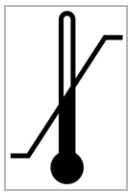
The following kits are available from Illumina for performing the Nextera DNA Flex Library Prep workflow.

Component	Kit Options	Catalog #
Library Prep	Nextera DNA Flex Library Prep (24 Samples)	20018704
	Nextera DNA Flex Library Prep (96 Samples)	20018705
Indexes	IDT for Illumina– Nextera DNA UD Indexes Set A (96 Indexes, 96 Samples)	20027213
	Nextera DNA CD Indexes (24 Indexes, 24 Samples)	20018707
	Nextera DNA CD Indexes (96 Indexes, 96 Samples)	20018708
Blood Lysis	Flex Lysis Reagent Kit (96 samples)	20018706
Additional Reagents (Optional)	Illumina Adapter Blocking Reagents (12 reactions)	20024144
	Illumina Adapter Blocking Reagents (48 reactions)	20024145
Additional Accessories for Nextera DNA CD Indexes (24 samples) (Optional)	Bag of 48 Index Adapter replacement caps, orange	15026585
	Bag of 32 Index Adapter replacement caps, white	15026586

Symbol Descriptions

The following table describes the symbols on the shipment packaging, consumable, or consumable packaging.

Symbol	Description
	Indicates the direction to the top of the box.
	Indicates that the contents are fragile and must be handled with care.

Symbol	Description
	Storage temperature range in degrees Celsius. Store the consumable within the indicated range.[1]
	The date the consumable expires. For best results, use the consumable before this date.
	Indicates the manufacturer (Illumina).
	The intended use is Research Use Only (RUO).
	Indicates the part number so that the consumable can be identified.[2]
	Indicates the batch code to identify the batch or lot that the consumable was manufactured in.[1]
	Indicates that caution is necessary.
	Indicates a health hazard.

[1] Storage temperature can differ from shipping temperature.

[2] REF identifies the individual component, while LOT identifies the lot or batch the component belongs to.

Nextera DNA Flex Library Prep

Box 1 of 3

These reagents are shipped at 2°C to 8°C. Promptly store reagents at the indicated tube temperature to ensure proper performance.

Tube Quantity		Reagent	Description	Storage Temperature
24 Samples	96 Samples			
1	1	SPB	Sample Purification Beads	2°C to 8°C
1	4	TSB	Tagment Stop Buffer	Room temperature
1	1	TWB	Tagment Wash Buffer	Room temperature

Box 2 of 3, Store at -25°C to -15°C

Tube Quantity		Reagent	Description
24 Samples	96 Samples		
1	1	RSB	Resuspension Buffer
1	4	TSB	Tagmentation Buffer 1
1	4	TWB	Enhancement PCR Mix

Box 3 of 3, Store at 2°C to 8°C

Tube Quantity		Reagent	Description
24 Samples	96 Samples		
1	4	BLT	Bead-Linked Transposomes

Index Kit Contents

Compatible index adapters are sold separately. The number of index adapter plates required for the Nextera DNA Flex Library Prep workflow depends on the number of samples to be uniquely indexed for your experiment.

The IDT for Illumina-Nextera DNA UD Indexes use 10 base pair index codes that differ from the Nextera DNA CD Indexes, which use 8 base pair index codes. This change in base pair index length may require adjustments to your sequencing run set up. For more information on sequencing run set up adjustments, see Unique Dual Indexes on the *Nextera DNA Flex Library Prep support page*.

These index kits are not interchangeable with the Nextera XT Index Kits.

Description
IDT for Illumina-Nextera™ DNA UD Indexes Set A (96 Indexes, 96 Samples)
Nextera DNA CD Indexes (24 Indexes, 24 Samples)
Nextera DNA CD Indexes (96 Indexes, 96 Samples)

Nextera DNA CD Indexes (24 Indexes, 24 Samples) Tube Format, Store at -25°C to -15°C

Quantity	Index Name	Description
1	H503	DNA Adapter
1	H505	DNA Adapter
1	H506	DNA Adapter
1	H517	DNA Adapter
1	H710	DNA Adapter
1	H705	DNA Adapter
1	H706	DNA Adapter
1	H707	DNA Adapter
1	H711	DNA Adapter
1	H714	DNA Adapter

IDT for Illumina Nextera DNA UD Indexes or Nextera DNA CD Indexes (96 Indexes, 96 Samples) Plate Format, Store at -25°C to -15°C

Quantity	Description
1	96 Dual Adapter Index Plate

Flex Lysis Reagent Kit (Optional)

These reagents are shipped at -25°C to -15°C. Promptly store reagents at the indicated tube temperature to ensure proper performance.

To start the protocol from fresh whole blood, the Flex Lysis Reagent Kit is required.

Quantity	Acronym	Description	Storage Temperature
4	BLB	Blood Lysis Buffer	15°C to 30°C
4	PK1	Proteinase K	-25°C to -15°C

NOTE

Sample Purification Beads are not included in this kit, however sufficient SPB to run the blood lysis workflow are included in the 24 Sample and 96 Sample Nextera DNA Flex Library Prep kits.

Consumables and Equipment

Confirm that all required user-supplied consumables and equipment are present and available before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
10 µl pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
20 µl pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl pipette tips	General lab supplier
1000 µl single channel pipettes	General lab supplier
96-well 0.8 ml Polypropylene Deepwell Storage (midi plate)	Thermo Fisher Scientific, catalog # AB-0859
Hard-Shell 96-well PCR plates	Bio-Rad, catalog # HSP-9601
1.7 mL Microcentrifuge Tubes	General lab supplier
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001
Microseal 'F' foil seals	Bio-Rad, catalog # MSF-1001
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, catalog # 89094-658
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, product # E7023
Nuclease-free water	General lab supplier
Qubit dsDNA HS Assay Kit	One of the following, depending on quantification method: • ThermoFisher Scientific, catalog # Q32851 • ThermoFisher Scientific, catalog # Q32854
Quant-iT™ PicoGreen® dsDNA Assay Kit	ThermoFisher Scientific, catalog # P11496
Qubit Assay Tubes	ThermoFisher Scientific, catalog # Q32856
One of the following kits, depending on quantification method: • [Fragment Analyzer] High Sensitivity NGS Fragment Analysis Kit • [Bioanalyzer] Agilent High Sensitivity DNA Kit	One of the following suppliers, depending on instrument: • Advanced Analytical, catalog # DNF-474 • Agilent, catalog # 5067-4626

Consumables for Blood and Saliva Input

Consumable	Supplier
[Blood] Flex Lysis Reagent Kit	Illumina, catalog # 20015884
[Blood] EDTA Blood Collection tubes	Becton Dickinson
[Saliva] Oragene DNA Collection Kit for Saliva	DNA Genotek, catalog # OGR-500 or OGD-510

Equipment

Equipment	Supplier
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge	General lab supplier
Microcentrifuge	General lab supplier
Vortexer	General lab supplier
Qubit® Fluorometer 3.0	ThermoFisher Scientific, catalog # Q33216, Q33217 or Q33218
One of the following analyzers: Advanced Analytical: • Fragment Analyzer™ Agilent Technologies: • 2100 Bioanalyzer Desktop System	Advanced Analytical: • See web product pages for catalog numbers Agilent Technologies: • Part # G2940CA
[Saliva] Water bath or air incubator reaching 50°C	As recommended by DNA Genotek, see product pages.

Thermal Cyclers

The following table lists the recommended settings for the thermal cycler. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad C-1000 Touch thermal cycler	Calculated	Heated	Plate
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated	Polypropylene plates and tubes
MJ Research DNA Engine Tetrad	Calculated	Heated	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

Acronyms

Acronym	Definition
BLB	Blood Lysis Buffer
BLT	Bead Linked Transposome
CD	Combinatorial Dual
EPM	Enhanced PCR Mix
EtOH	Ethanol
PK1	Proteinase K
RSB	Resuspension Buffer
SPB	Sample Purification Beads
TB1	Tagmentation Buffer 1
TSB	Tagment Stop Buffer
TWB	Tagment Wash Buffer
UD	Unique Dual

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.066.5835	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
France	+33 805102193	+33 170770446
Germany	+49 8001014940	+49 8938035677
Hong Kong	800960230	
Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
Norway	+47 800 16836	+47 21939693
Singapore	+1.800.579.2745	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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Illumina
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

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